Original Article

Hydrogen-enriched water eliminates fine particles from the lungs and blood by enhancing phagocytic activity

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Abstract

Particulate matters (PM) are one of the major body burdens leading to diseases. We investigated the capacities of a hydrogen-enriched water (HW) eliminating carbon nanoparticles (CNP) and carbon microparticles (CMP) from the lungs and blood, respectively. In CNP-elimination test, rats were orally administered with purified water (PW) or HW (10 or 30 mL/kg/day) for 10 weeks. At the time point of 4 weeks, the rats were challenged with intratracheal instillation of CNP (4 mg). CNP accumulated in the airways and alveoli, and induced inflammatory lesions. Such pneumoconiosis was markedly improved by feeding HW, while PW was ineffective. CNP-induced pneumoconiosis caused systemic hematological alterations, decreasing major inflammatory cells, but markedly increasing eosinophils, indicative of an allergic reaction, which were attenuated by treatment with HW. Such PM-eliminating and anti-allergic effects of HW reduced body burden as confirmed from the facilitated recovery of body and lung weights. In CMP-clearance test, mice were orally administered with PW or HW for 7 days, and intravenously injected with CMP (300 mg/kg). CMP was rapidly eliminated from the blood in HW-fed mice. Indeed, the phagocytic indices increased to 3.5 and 6.7 folds at 10 and 30 mL/kg of HW, in comparison with a negligible effect of PW. As a mechanism study, only HW significantly inhibited lipid peroxidation *in vitro* Fenton reaction-mediated ·OH-generating system. Collectively, the results indicate that HW not only effectively eliminated PM from the lungs and blood by enhancing phagocytic activity, but also attenuated the lung injuries by inhibiting lipid peroxidation.

Keywords: particulate matter, pneumoconiosis, hydrogen-enriched water, particle clearance, antioxidation

Introduction

Air pollution is associated with problems to human health as well as loss of quality of life[1–3]. Air pollutants fall into two categories: gases (for example, O_3, NO_2, SO_2, CO, and so on) and particulate matters (PM), with different grain sizes and chemical composition. These pollutants have been linked to adverse health effects even in low concentrations, especially PM, the most responsible for health problems related to the respiratory system[4–5]. The rapid industrialization of the earth has been inducing desertification and

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environmental pollution, and thereby increasing exposure of humans and wildlife population to a lot of fine particles such as industrial particulates and sands. The fine particle exposure is one of the major burdens of disease attributable to 20 leading risk factors, and especially, it is in higher rank in East Asian countries such as China and Korea[6]. The harmful effect to human health caused by PM depends both on its concentration in the inhaled air and on its granulometry, and chemical composition.

The PM is classified according to its aerodynamic diameter (varies from few nanometers to 100 μm). In addition to sedimentary particles (SP) that cause discomfort, the inhalable PM harmful to health are divided into fine particles smaller in diameter than 10 μm (PM10) and ultrafine particles smaller than 2.5 μm (PM2.5)[5,7]. The harmful effects of PM2.5 occur in the short-term, *via* direct action on the airways, as well as in the long-term, leading to systemic adverse effects, because the particles can reach the alveoli, enter the bloodstream, and affect other organs besides the lungs[8]. Thus, PM2.5 presents potential health risk even when in relatively low concentrations in the atmosphere; i.e., even when its atmospheric concentration is below the maximum levels of tolerance established by the World Health Organization (WHO) and by the main environmental regulatory agencies of the world[5,8].

Studies show the association between air pollution and incidence of respiratory, cardiovascular, neurological diseases, and several types of cancer[5,7,9]. The association with respiratory diseases is stronger and direct, and the most vulnerable groups are children, elderly people, and those with pre-existing respiratory diseases, especially asthma, chronic bronchitis, and obstructive pulmonary disease[2–3,10–12]. PM exposure triggers a variety of maladaptive signaling pathways in the lungs, blood vessels, liver, and brain that are associated with endoplasmic reticulum (ER) stress, oxidative stress, and inflammatory responses[13–15]. Oxidative stress arises from the strong cellular oxidizing potential of excess reactive oxygen species (ROS), or free radicals[16–17]. The ROS, generated *via* diverse physiologic and pathological activities, is converted to highly toxic hydroxyl group (·OH) or detoxified by oxidizing and antioxidant enzymes, respectively. High concentration of ·OH is produced *via* Fenton reaction mediated by transition metal ions such as Fe^{2+} and Cu^{2+}. Since there is no known detoxification system for ·OH, scavenging ·OH is a critical antioxidant process[18–19].

Molecular hydrogen (dihydrogen, H_2) has been suggested that it acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals[18]. Indeed, hydrogen water has been reported to improve various diseases and tissue injuries through anti-oxidative and anti-inflammatory activities including pulmonary inflammation and asthma[20–24], cerebral infarction (stroke)[18], Alzheimer disease[25], Parkinson disease[26], rheumatoid arthritis (RA)[27], and diabetes[28]. Notably, in addition to tissue-protective effects, hydrogen water attenuated the brain inflammation induced by particulate matters[15]. Such multifaceted beneficial effects of hydrogen water led us to investigate its effectiveness on the fine particle burden in the lungs and blood, and underlying mechanisms.

Materials and methods

Materials

Hydrogen-enriched water (HW) was obtained from Anydoctor Healthcare Co., Ltd. (Cheonan, Korea). To produce HW, filtered purified water (PW) with 99% transmittance was nitrogenized in aluminum cans, and infused with H_2 gas under a pressure of 0.2 Mpa using a Hydrogen manufacturing device (Sang-IL Co., Ltd, Geochang, Korea). The can rapidly seamed and sterilized at 75°C (central temperature) for 10 minutes. H_2 concentration in HW was confirmed to be 1.2–1.4 ppm (pH 7.3–7.9). Carbon nanoparticles (CNP; ⩽500 nm, CAS 1333-86-4) and carbon microparticles [CMP (Speedball superblack India ink) ⩽10 μm] were procured from Sigma-Aldrich (St. Louis, MO, USA) and Homi Art Shop (Seoul, Korea), respectively.

Animals

Six-week-old male Wistar Hannover rats and 6-week-old male ICR mice were obtained from Daehan Biolink (Eumseong, Korea). The animals were housed in a room with a constant temperature (23±2°C), relative humidity (55±10%), and a 12-h light/dark cycle. The animals were fed standard rodent diet (Hanlan #2018) and purified water *ad libitum*. All experimental procedures were carried out in accordance with the Standard Operation Procedures of Laboratory Animal Center, Chungbuk National University (CBNU), Korea. The protocol was approved by the Institutional Animal Care and Use Committee of CBNU.

Elimination of ultrafine particles from the lungs

After 1-week acclimation to the laboratory environment, the rats (7 weeks old, n = 7/group) were orally administered twice a day with low (10 mL/kg/day) or high (30 mL/kg/day) doses of purified water (PW) or HW for 10 weeks. At time point of 4 weeks from the

initial treatment during the 10-week feeding period, the rats in treatment groups were challenged with intra-tracheal instillation of CNP. In brief, a particle dispenser was made by fixing a 5 cm 4/0 monofilament nylon suture in 2 layers of 5 cm narrow polyethylene tube A (0.61 cm in diameter) and 2 cm wide tube B (1.45 cm in diameter). One end of the aligned 3 layers (nylon suture and tubes A and B) was flame-sealed, and 4 holes were punctured through the outer wide tube B at their flank using a syringe needle. The narrow tube A including nylon suture was inserted into a Sonde needle connected to a syringe containing CNP (**Supplementary Fig. 1**). CNP (4 mg) was suspended in 150 μL PG-CMC (10% propylene glycol and 1% sodium carboxymethyl cellulose in saline), and instilled into the rat's trachea through the prefixed dispenser guided by a small animal laryngoscope (LS-2-R; Penn-Centry Inc., Wyndmoor, USA).

During the whole experimental period, bodyweights were recorded. At the end of the experiment, the rats were sacrificed and blood samples were collected from the abdominal aorta, and complete blood counts (CBC) were analyzed. Lungs were collected, weighed, and fixed in a neutral formalin solution. Paraffin-embedded lung sections were stained with hematoxylin-eosin, and examined under a light microscope for CNP accumulation and lesions. The accumulation and lesions were scored according to the area and severity with a maximum score of 10.

Clearance of fine particles from the blood

After 1-week acclimation to the laboratory environment, the mice (7 weeks old, $n = 5$/group) were orally administered twice a day with low (10 mL/kg/day) or high (30 mL/kg/day) doses of purified water (PW) or HW for 7 days. Thirty min after the final treatment, the mice were intravenously injected with CMP. CMP was centrifuged at 5,000 rpm for 15 minutes. Then, the supernatant was diluted 3 folds with sterile 1.5% gelatin in saline to adjust to 30 mg/mL. The diluted carbon suspension was intravenously injected at a volume of 10 mL/kg (300 mg/kg). After 0.5 and 10 minutes of CMP injection, 50 μL blood sample was collected, hemolyzed by adding 1 mL Na_2CO_3 (0.1%) solution. The absorbance was measured at 600 nm, and the phagocytic index (K) was calculated from an equation: $K = 1/t_{10} − t_{0.5} \times \log(C_{0.5} − C_{10})$.

Antioxidation *in vitro*

Saline-perfused lung tissue was collected, and homogenized in cold 10 mmol/L phosphate-buffered saline (PBS, pH 7.4) to make 10% homogenate. To detect antioxidative capacity, PW and HW (final concentrations of 10% and 20%) were added to PBS containing 10% lung homogenate, and 0.1 mmol/L ferrous perchlorate (0.1 mmol/L). Fenton reaction was initiated by adding H_2O_2, and incubated at 37°C for 30 minutes. Into the tissue homogenate (250 μL), 250 μL sodium dodecyl sulfate (SDS, 8.1% solution) and 500 μL 20% acetic acid (adjusted to pH 3.5) were added. After adding 250 μL 2-thiobarbituric acid (TBA, 0.75% solution), the mixture was boiled in a glass tube capped with aluminum foil for 30 minutes. Samples were cooled on ice, centrifuged at 13,000 g for 10 minutes, and absorbance of the supernatant was read at 532 nm for the quantification of TBA-reactive substances (TBARS).

Statistical analysis

The results are displayed the means±standard error. The significance of differences of all results was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's (LSD) test, using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set a priority at $P < 0.05$.

Results

Intratracheal challenge with CNP (4 mg) decreased the rats' bodyweights for 2-3 days, indicative of impact and burden of pulmonary pneumoconiosis (**Fig. 1**). Notably, daily feeding of PW and HW (10 or 30 mL/kg) facilitated the recovery of bodyweights to some extent. In contrast to the decrease in bodyweights, the absolute and relative lung weights increased following CNP inhalation (**Table 1**). However, treatment with HW
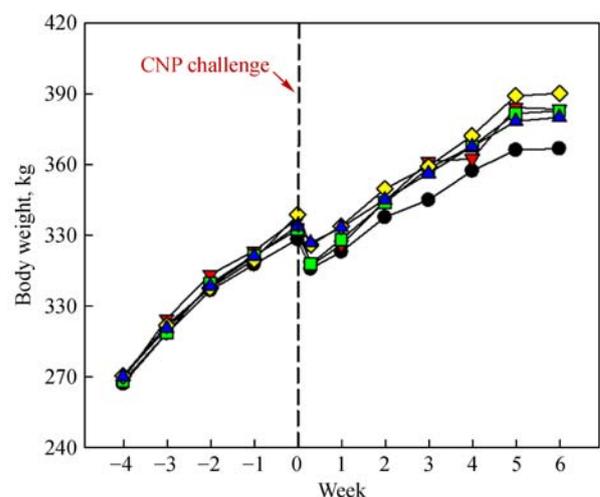

Fig. 1 Time-course of mean bodyweights of rats fed purified water (PW) or hydrogen-enriched water (HW). ●: carbon nanoparticle (CNP) alone, ▼: + 10 mL/kg PW, ■: + 30 mL/kg PW, ♦: + 10 mL/kg HW, ▲: + 30 mL/kg HW.

Table 1   **Absolute and relative lung weights of rats fed purified or hydrogen-enriched water 6 weeks after carbon nanoparticle instillation**

Treatment (mL/kg)	Body weight (g)	Absolute lung weight (g)	Relative lung weight (%)
Normal	370.1±15.2	2.35±0.10	0.64±0.03
CNP	366.8±5.7	2.62±0.20	0.74±0.06
+ PW (10)	383.5±20.0	2.59±0.13	0.65±0.03
+ PW (30)	382.9±15.9	2.55±0.09	0.69±0.03
+ HW (10)	390.3±20.5	2.43±0.22	0.60±0.03*
+ HW (30)	380.0±7.9	2.40±0.07	0.63±0.01*

CNP: carbon nanoparticles, PW: purified water, HW: hydrogen-enriched water. *Significantly different from CNP alone ($P < 0.05$).
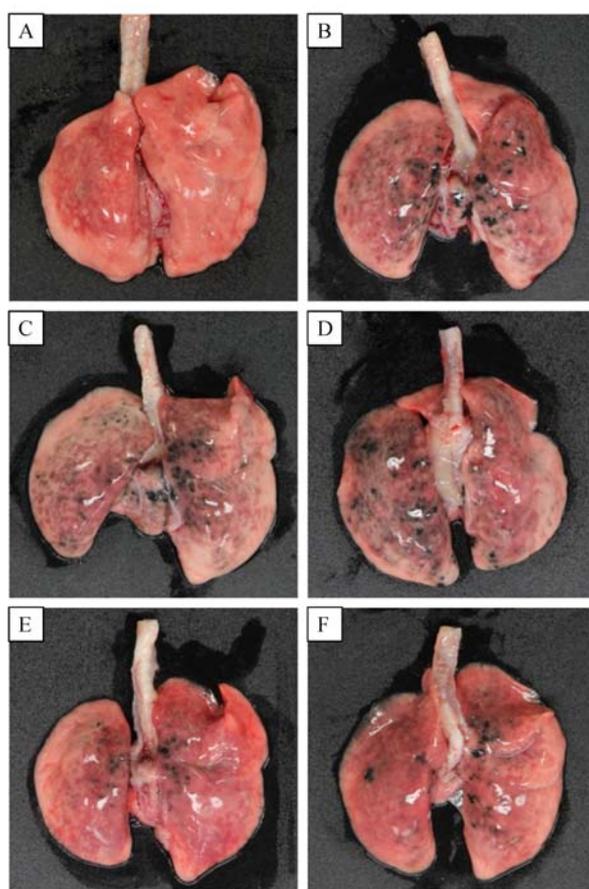

Fig. 2   **Gross findings of the lungs of rats fed purified water (PW) or hydrogen-enriched water (HW) 6 weeks after carbon nanoparticle (CNP) instillation.** A: Normal; B: CNP; C: PW10: 10 mL/kg PW; D: PW30: 30 mL/kg PW; E: HW10: 10 mL/kg HW; F: HW30: 30 mL/kg HW.

significantly attenuated CNP-induced increase in lung weights, in which HW was superior to PW.

In gross findings, intratracheal instillation of CNP caused serious pneumoconiosis, resulting in overall accumulation of the ultrafine particles in the lungs (*Fig. 2*). Severe accumulation of CNP in the airways and parenchymal tissues, and ensuing tissue injuries were confirmed in microscopic observations (*Fig. 3*). Interestingly, the accumulation of carbon particles as

well as inflammatory lesions were markedly eliminated by feeding HW in a dose-dependent manner, although the effect of PW was negligible (*Fig. 4*).

CNP-induced pneumoconiosis caused systemic hematological alterations (*Table 2*). CNP exposure significantly decreased white blood cells (WBC), monocytes, and platelets, but markedly increased eosinophils, indicative of an allergic reaction in spite of general immunosuppression. Notably, PW and HW
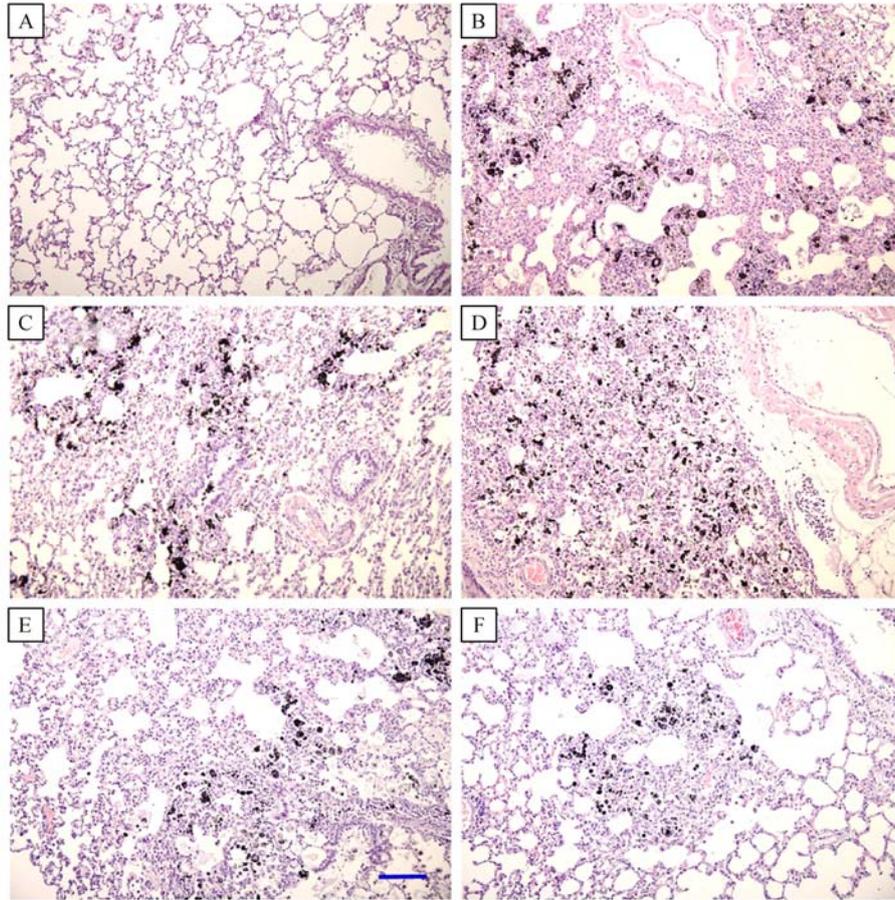
Fig. 3 Microscopic findings of the lungs of rats fed purified water (PW) or hydrogen-enriched water (HW) 6 weeks after carbon nanoparticle (CNP) instillation. A: Normal; B: CNP; C: PW10: 10 mL/kg PW, D: PW30: 30 mL/kg PW, E: HW10: 10 mL/kg HW, F: HW30: 30 mL/kg HW. Bar = 100 μm.

remarkably attenuated the increase in eosinophils induced by CNP, and restored the number of platelets to some extent, although HW was superior to PW in regulating the eosinophil reaction.

Intravenously injected CMP was slowly eliminated from the blood in normal mice (*Fig. 5A*). The elimination velocity was slightly increased by pretreatment with PW, doubling the phagocytic index at a high dose (30 mL/kg) (*Fig. 5B*). Notably, HW was superior to PW, increasing phagocytic indices to 3.5 and 6.7 folds at 10 and 30 mL/kg, implying that HW enhanced phagocytic activity of blood macrophages.
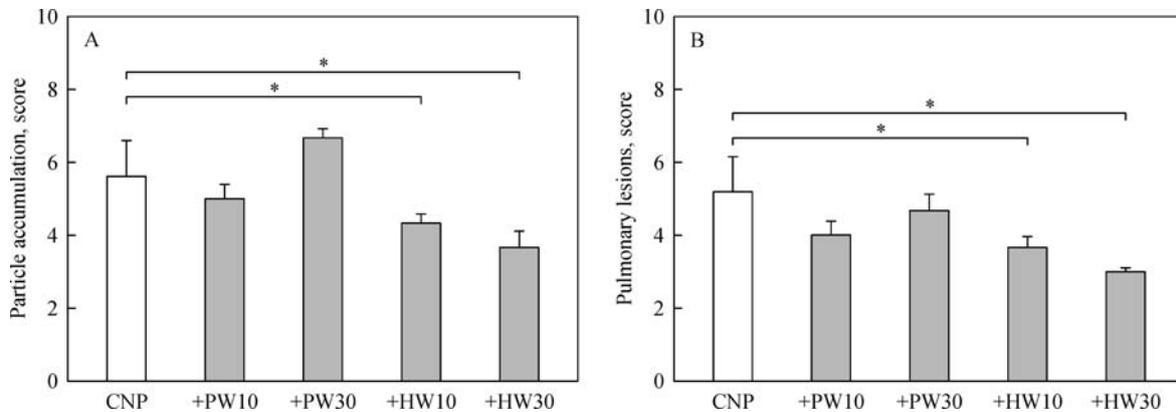

Fig. 4 Scores of carbon nanoparticle accumulation (A) and pulmonary lesions (B) of the lungs of rats fed purified water (PW) or hydrogen-enriched water (HW) 6 weeks after carbon nanoparticle (CNP) instillation. PW10: 10 mL/kg PW, PW30: 30 mL/kg PW, HW10: 10 mL/kg HW, HW30: 30 mL/kg HW.

Table 2 **Hematology of rats fed purified or hydrogen-enriched water 6 weeks after carbon nanoparticle instillation**

Treatment (mg/kg)	Normal	CNP	+ PW (10)	+ PW (30)	+ HW (10)	+ HW (30)
WBC (10^3/μL)	5.56±1.52	3.95±0.32*	4.42±0.38	5.13±0.62	4.43±0.52	4.02±0.63
Neutrophils (%)	11.10±2.72	15.40±2.01	19.15±1.46	14.58±1.14	16.40±3.10	18.34±2.59
Basophils (%)	0.11±0.06	0.07±0.03	0.20±0.07	0.13±0.03	0.20±0.07	0.08±0.04
Eosinophils (%)	0.96±0.64	4.17±1.97*	3.75±1.18	2.30±0.32	1.85±0.21[#]	2.04±0.60[#]
Lymphocytes (%)	83.49±3.24	79.30±2.25	75.95±2.58	81.93±1.09	80.63±3.21	78.66±3.16
Monocytes (%)	1.71±0.61	1.07±0.15*	0.95±0.18	1.08±0.24	0.93±0.10	0.88±0.11
Platelets (10^3/μL)	1,384.6±137.4	735.3±40.5*	776.0±83.3	973.8±25.6	789.5±40.0	834.8±60.1
RBC (10^6/μL)	7.90±0.25	8.37±0.36	8.39±0.24	8.40±0.18	8.19±0.15	8.22±0.47

CNP: carbon nanoparticles, PW: purified water, HW: hydrogen-enriched water. *Significantly different from Normal ($P < 0.05$). [#]Significantly different from CNP alone ($P < 0.05$).

TBARS concentration in the lung tissue greatly increased in a Fenton reaction-mediated ·OH generating condition (*Fig. 6*). Such lipid peroxidation was significantly lowered by treatment with HW in a concentration-dependent manner, but not by PW, suggestive of the antioxidative capacity of HW.

Discussion

It is well known that the types of pneumoconiosis are depending upon the kinds of causative particles such as environmental dusts including Yellow sands, coals and carbons (coal workers' pneumoconiosis), asbestos (asbestosis, malignant mesothelioma, and lung cancer), silica (silicosis), iron (siderosis), etc[29–30]. These particles agglomerate in lung alveolar cells. Coal workers' pneumoconiosis is a chronic occupational and a restrictive lung disease caused by the inhalation of carbon particles into the alveoli. Microorganisms as well as PM including carbon particles in the airways are removed by alveolar macrophages[31], during which proinflammatory cytokines and ROS are released from the activated macrophages[32–33]. However, a heavy burden of PM destroys macrophages, and inflammatory cytokines and ROS have potentials to induce lung tissue injury[31,33].

We observed serious accumulation of CNP in the airways and parenchyma as well as pulmonary lesions following intratracheal instillation of the particles. However, the pneumoconiosis was remarkably attenuated by a long-term feeding of HW, but not by PW. Notably, the CNP was markedly removed by HW, suggestive of an enhanced phagocytic activity of alveolar macrophages. Such facilitated clearance of CNP from the lungs by HW was also confirmed from the blood clearance of CMP: i.e., pretreatment with HW greatly enhanced the phagocytic index of blood
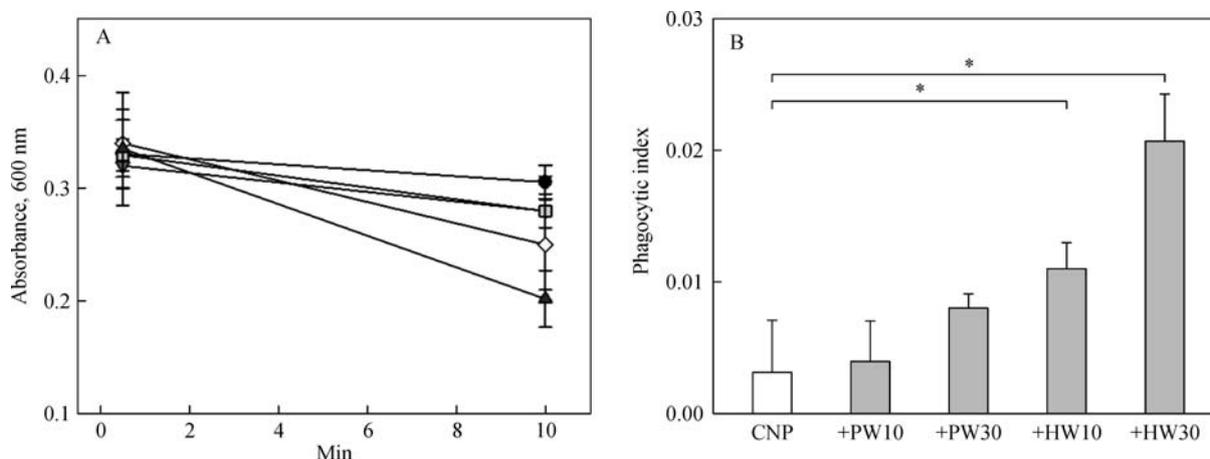

Fig. 5 **Clearance of carbon microparticles (CMP) from blood of mice fed purified water (PW) or hydrogen-enriched water (HW) during 10 minutes after carbon CMP injection.** A: Elimination of CMP, ●: CMP alone, ▼: + 10 mL/kg PW, ■: + 30 mL/kg PW, ♦: + 10 mL/kg HW, ▲: + 30 mL/kg HW. B: Phagocytic index. PW10: 10 mL/kg PW, PW30: 30 mL/kg PW, HW10: 10 mL/kg HW, HW30: 30 mL/kg HW.
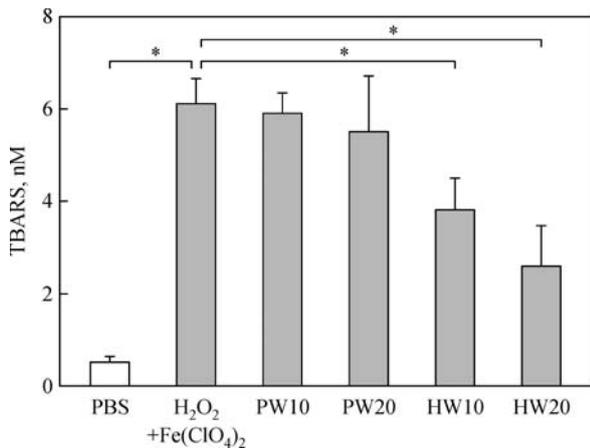
Fig. 6 **Antioxidative activity of purified water (PW) or hydrogen-enriched water (HW) against lipid peroxidation induced by hydrogen peroxide (H_2O_2) plus ferrous perchlorate [$Fe(ClO_4)_2$].** PW10: 10% PW, PW20: 20% PW, HW10: 10% HW, HW20: 20% HW.

macrophages, in comparison with negligible effect of PW. Recent study suggested that liver-resident macrophages, or Kupffer cells, are key cells in the hepatic sequestration of nanoparticles[34]. The authors showed that human macrophage phenotype modulates hard nanoparticle uptake, in which "regulatory" M2 phenotype human macrophages, rather than "inflammatory" M1 phenotypes, preferentially took up gold nanoparticles, with a clear hierarchy among the subtypes (M2c > M2 > M2a > M2b > M1). Additional studies are needed to explain mechanism(s) and macrophage-

phenotype specificity for the effectiveness of HW in the CNP and CMP clearance.

It is believed that CNP induced pulmonary lesions via direct injury as well as indirect inflammation mediated by cytokines and ROS from activated alveolar macrophages[31–33]. It is of interest to note that in spite of the enhanced phagocytic activity in the lungs and blood following HW feeding, the lung injury induced by CNP instillation was attenuated by HW, which may be due to its strong antioxidative potential as confirmed by the suppressive activity on Fenton reaction-mediated lipid peroxidation. Such antioxidant activity of HW is supported by previous reports demonstrating its suppressive efficacies on paraquat- and irradiation-induced lung injuries[22–23]. In addition, HW displayed anti-inflammatory activities, attenuating cigarette smoke-induced airway mucus production[21] and regulating proinflammatory and anti-inflammatory cytokines in lipopolysaccharide-stimulated RAW 264.7 macrophages[35]. Interestingly, HW did not inhibit multiple points of the cascade of radical reactions other than the final stage producing $\cdot OH$ group[18]. Therefore, it is proposed that HW not only eliminates the CNP by activating alveolar macrophages, but also attenuates lung injury *via* direct antioxidative potential masking the activated macrophage-mediated inflammatory stress, in dual manners.

As aforementioned, HW reduced the burden of CNP accumulation in the lungs, and thereby facilitated the recovery of bodyweight and lung weight of the CNP-challenged animals. It is also believed that elimination by HW of CNP from the lungs should block further long-term inflammation and tissue injury. Such
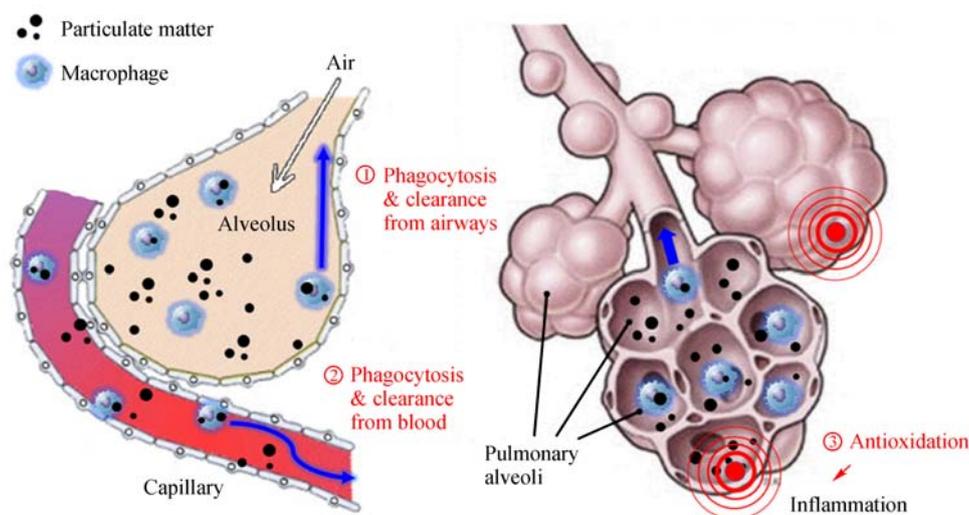

Fig. 7 **Schematic summary of the fine particle-eliminating and tissue-protecting efficacies of hydrogen-enriched water**: enhanced phagocytosis and elimination from the airways (①), phagocytosis and elimination from the blood (②), and anti-inflammation *via* antioxidative potential (③).

enhanced clearance of PM from the lungs and blood might be related to the hematological changes. Actually, CNP challenge decreased the major defensive inflammatory cells (WBC, monocytes, and platelets), but increased eosinophils, indicative of general immune dysfunction and sensitized allergic reaction. In particular, intrapulmonary infusion of CNP in healthy males significantly increased the number of WBC, especially neutrophils, 6 hours later[36]. In our long-term (6 weeks) study, there was a light increase in the ratio of neutrophils. The adverse effects of PM on host defense system were in part, especially on eosinophil response, restored by treatment with HW. Such anti-allergic activity of HW was confirmed in asthma, atopic dermatitis, and RA animals[24,27,37–38].

Taken together, HW not only effectively eliminated both the ultrafine and fine particles from the lungs and blood by enhancing phagocytic activity, but also attenuated the lung tissue injuries by inhibiting lipid peroxidation. Such multifaceted activities of HW on the PM elimination and tissue protection was summarized in ***Fig. 7***, suggesting enhanced phagocytosis and elimination from the airways (①) and blood (②) and anti-inflammation *via* antioxidative potential (③). The results indicate that that a long-term intake of HW might be beneficial for the reduction of bodily burden of PM.

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